Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus become vasopressin positive after adrenalectomy

(colocalization/parvocellular neurons/hypothalampituitary adrenocortical regulation)

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ABSTRACT The immunoperoxidase technique was used to study the effect of adrenalectomy on vasopressin (VP) immunoreactivity in the hypothalamic paraventricular nucleus of rat. In control animals, relatively few VP-immunostained parvocellular neurons were found in addition to a large population of magnocellular VP neurons. Seven to 14 days after bilateral adrenalectomy, VP immunostaining increased markedly in specific subdivisions of the paraventricular nucleus. In contrast to normal animals, VP immunoreactivity was localized in a large number of parvocellular neurons. Colchicine treatment, on the other hand, did not significantly increase the number of VP-immunostained parvocellular neurons found in control rats. These observations suggest that adrenalectomy increases the number of VP-positive neurons and appears to increase the intensity of VP immunoreactivity specifically in parvocellular neurons. VP parvocellular neurons are confined to those paraventricular nucleus subdivisions that are known to project to the external zone of the median eminence. Moreover, their distribution pattern is very similar, if not identical, to that of the corticotropin-releasing factor (CRF) immunoreactive cells. Parvocellular neurons on adjacent thin sections could be stained for both CRF and VP. Thus, adrenalectomy seems to increase VP staining in CRF immunoreactive parvocellular neurons, which innervate the external zone of the median eminence.

Vasopressin (VP) participates in the regulation of corticotropin secretion, potentiating the action of corticotropin-releasing factor (CRF) on the pituitary (1–6). In support of this concept, anatomical studies have shown the presence of a VP fiber system capable of delivering a considerable amount of this peptide to the adenohypophysis (7–10). Neurophysin-containing fibers and varicosities have been described in the vicinity of the portal capillary plexus of the external zone of the median eminence (ME) (5, 10, 11). It is clear now that the majority of these neurophysin-positive axons are vasopressinergic (12, 13). Lesion studies indicated that the source of VP to the external zone is the hypothalamic paraventricular nucleus (14, 15), although the exact cellular origin of these fibers was unclear.

A particularly interesting feature of this system is that adrenalectomy substantially increased VP immunoreactivity in the external zone (13, 15, 16). This increase could be blocked by glucocorticoid but not by mineralocorticoid treatment (13, 17, 18). Furthermore, 5 days of dehydration did not affect VP immunostaining in the external zone (13, 19). Thus, the increase of VP immunoreactivity after adrenalectomy seems to be due to the lack of glucocorticoid feedback rather than a disturbance in fluid homeostasis. These observations support the hypothesis that VP is involved in the regulation of the pituitary–adrenal axis.

In the present study, we sought to determine whether the increased VP immunoreactivity in the external zone after adrenalectomy is accompanied by changes in VP immunostaining in certain subdivisions of the paraventricular nucleus. We found that adrenalectomy substantially increased VP staining in parvocellular neurons, which also contain CRF.

METHODS AND MATERIALS

Male Sprague–Dawley rats weighing 200–250 g were kept under standard laboratory conditions; 12–12 hr light/dark cycle, standard rat chow pellets, and tap water ad lib. Three groups of rats were examined. (i) Normal untreated animals. (ii) Colchicine-treated animals. To increase immunostaining of cell bodies, colchicine (Sigma) was injected into the lateral ventricle (50 μg dissolved in 25 μl of 0.9% NaCl) 48 hr prior to sacrifice. (iii) Animals subjected to bilateral adrenalectomy. Bilateral adrenalectomy was carried out under ether anesthesia using a dorsal approach. After the operation, animals were allowed free access to food and 0.9% NaCl as well as tap water.

Tissue Preparation and Immunohistochemistry. Adrenalectomized animals survived 7–14 days. Rats were anesthetized and their brains were fixed by transaortic perfusion of ice-cold (4°C) 4% paraformaldehyde/0.2% picric acid/0.167 M sodium phosphate buffer, pH 7.0. The brains were cut into blocks, which were placed in ice-cold fixative for 3 hr and then washed in several changes of sodium phosphate buffer (pH 7.2). Thick (20–50 μm) coronal sections were cut with a vibratome (Oxford) and processed for immunohistochemistry. Sections were incubated with CRF or VP antiserum (diluted 1:500 and 1:1000, respectively, in sodium phosphate buffer, pH 7.2) at 4°C for 24–48 hr, washed in sodium phosphate buffer, incubated with biotinylated anti-rabbit IgG (1:250) for 1 hr, and placed with avidin-biotin-peroxidase complex (ABC) (90 μl of avidin/90 μl of biotinylated peroxidase to 10 ml of buffer) for 1 hr. The ABC components were obtained in kit form (Vectastain ABC kits, Vector Laboratories, Burlingame, CA). The chromogen used was 3,3-diaminobenzidine tetrahydrochloride (Hach, Ames, IA). All solutions contained 0.1% Triton X-100 (Mallinckrodt). Sections were mounted on chromium-coated slides, dehydrated, and coverslips were placed over them.

To colocalize CRF and VP immunoreactivity, 5-μm thin cryostat sections were cut and processed for indirect immunofluorescence histochemistry. Briefly, adjacent sections were incubated with the antiserum to CRF (1:400 dilution) or

Abbreviations: CRF, corticotropin-releasing factor; ME, median eminence; VP, vasopressin.
to VP (1:800), rinsed in 0.1 M sodium phosphate buffer (pH 7.2), incubated with fluorescein isothiocyanate-conjugated antibodies, rinsed again, mounted, examined with a Zeiss fluorescence microscope, and photographed.

Antisera and Controls. VP antiserum was provided by James Russell (National Institute of Mental Health). The antibody was raised in rabbits against arginine-VP conjugated to bovine serum albumin via glutaraldehyde, and it binds to the COOH-terminal part of the molecule. For the binding, the VP must be amidated. The antibody does not crossreact with oxytocin, neurophysin, or the precursor (propressophysin) molecule. It has a 100% crossreactivity with arginine-vasotocin. Specificity of antiserum was tested by preincubation of the antisemur with synthetic arginine-8-vasopressin peptide (Sigma) (10 μg in 1 ml of diluted antiserum). No immunostaining was detected after the preabsorption. Preabsorption of the antiserum with 10 μg of CRF per ml (Peninsula Laboratories, San Carlos, CA) did not affect VP staining intensity.

Antiserum against CRF was obtained from Robert Eskay (National Institutes of Health). It was produced in rabbits against ovine CRF (Peninsula) conjugated to bovine thyroglobulin via glutaraldehyde. Immunostaining was abolished after preabsorption with 10 μg of CRF per ml. No changes were observed in immunostaining after incubation of the antiserum with 10 μg of arginine-8-vasopressin per ml.

RESULTS
In the present study, we have used nomenclature for paraventricular nucleus subdivisions introduced by Armstrong et al. (20), Swanson and Kuypers (15), and Sawchenko and Swanson (21).

Immunostaining of VP and CRF in the Paraventricular Nucleus of Normal and Colchicine-Treated Animals. In normal rats, the distribution of VP-immunoreactive neurons in the paraventricular nucleus was consistent with previous reports (21, 22). The large majority of positive cells were typical magnocellular neurons; a few parvocellular VP neurons were detected in the anterior, dorsal, and medial paraventricular subdivisions (Fig. 1). Immunostaining of these parvocellular neurons was lighter than staining of magnocellular neurons. Some large VP neurons were located in the parvocellular subdivisions. Colchicine treatment increased the intensity of VP immunostaining in both magno- and parvocellular neurons. In addition, there seemed to be a slight increase in the number of immunoreactive neurons. The distribution of VP among cells, however, remained unchanged. That is, a large number of magnocellular and a small number of parvocellular neurons were stained.

VP-positive fibers and varicosities were present in moderate to low density in the external zone. They were found throughout the entire rostrocaudal extent of the ME in normal and colchicine-treated animals.

In agreement with earlier studies (23, 24), only a few scattered CRF-containing neurons were visualized in the paraventricular nucleus of normal animals. Colchicine treatment substantially increased the staining intensity and the number of CRF-immunostained neurons. Stained neurons were confined to the anterior and medial paraventricular subdivisions.

A dense plexus of CRF-immunopositive fibers and terminals was observed in the external zone of the ME in normal animals.

Immunostaining After Adrenalectomy. Seven to 14 days after adrenalectomy, there was a substantial increase in VP immunostaining in the paraventricular subdivisions of the paraventricular nucleus over that seen in normal animals (Fig. 1). Adrenalectomy not only intensified immunostaining in these neurons but also markedly increased the number of parvocellular immunopositive neurons. These cells were concentrated in the anterior and medial parvocellular subdivisions. No increase of VP immunoreactivity was observed in the dorsal parvocellular and posterior magnocellular subdivisions projecting to the brainstem and spinal cord.

A dense arborization of VP fibers and varicosities was observed throughout the external zone of the ME of adrenalectomized rats.

The distribution and the approximate number of CRF-immunostained cell bodies after adrenalectomy were similar to those after colchicine treatment (Fig. 1).

Because the distributions of VP and CRF-immunoreactive neurons appeared similar, we attempted to colocalize the two substances in the same neuron. We found a considerable number of parvocellular neurons that contained both CRF and VP (Fig. 2). However, some of the neurons in the same parvocellular subdivisions appeared to contain only one or the other of these peptides.

DISCUSSION
We have confirmed the observation that VP is present in fibers and varicosities in the external zone of the ME and that removal of the adrenals increases VP staining in these fibers markedly (13, 16, 25). Earlier studies have led to the suggestion that magnocellular neurons provide both the external zone and the posterior pituitary with their vasopressinergic innervation (26-29). Our data show that this is not the case. Parvocellular neurons that also contain CRF seem to be the source of the VP-containing neuronal processes in the external zone. The evidence for this is (i) that parallel changes in VP staining in the external zone and paraventricular subdivisions occur in response to adrenalectomy; (ii) the VP-positive parvocellular neurons observed in adrenalectomized animals are confined to subdivisions of the paraventricular nucleus that are known to project to the ME (30, 31); (iii) VP immunoreactivity was localized in CRF-immunopositive parvocellular neurons, which have been reported to send axons to the external zone (23, 24). Thus, separate populations of VP neurons project to the ME and posterior pituitary. Furthermore, those VP neurons that innervate the ME do not send fibers to the brainstem and spinal cord.

The mechanism by which adrenalectomy induces an increase in VP staining in the parvocellular neurons remains unclear. It is clear, however, that the effect of adrenalectomy is quite specific. We observed that other peptide transmitters in paraventricular nucleus cells, such as cholecystokinin (32) and neurotensin (33), do not appear to be affected by adrenalectomy. It is apparent that adrenalectomy does not induce VP staining in all CRF-producing cells. In this regard, we observed that the CRF-positive neurons in the central nucleus of the amygdala (24), for instance, do not respond to adrenalectomy by making VP.

The anti-VP antibody used in our studies is COOH terminally directed and requires for binding that the peptide be amidated. It does not recognize the VP precursor, propressophysin (James Russell, personal communication). The increase in VP staining then should be due to an increase in VP itself in the cell bodies. Russell et al. (34) have reported that the rate of the synthesis of VP precursor increases in the paraventricular nucleus after adrenalectomy. Our data suggest that there may be a concomitant increase in precursor processing. The fact that we see little VP staining in the parvocellular population of neurons even after colchicine treatment indicates that the level of VP production in these cells must normally be very low.

The increase in VP staining that we have observed is surely an indication of a role for VP in regulation of adrenocorticotropic hormone release. Evidently, VP and CRF are secreted together in adrenalectomized animals and act in concert on the corticotrophs. This may or may not be the case in normal animals.
Fig. 1. Topography of VP (AVP)- and CRF-immunoreactive neurons in the paraventricular nucleus of normal (norm) and adrenalectomized (ADX) rats. Photomicrographs show coronal sections of the paraventricular nucleus at various levels; row A, 1700 μm; row B, 1900 μm; and row C, 2000 μm behind the bregma. The white dashed line outlines the parvocellular subdivision of the paraventricular nucleus. Note the large increase in number of VP parvocellular neurons in adrenalectomized rats (second column, white dashed line) over the relatively few immunostained parvocellular neurons of normal animals (first column) and the considerable overlap of the distribution of CRF (third column) and VP-containing parvocellular neurons. (×90.)
As AVP(ADX) goes...

...14 C

B. CRF(ADX)

FIG. 2. (A and B) Immunofluorescence micrographs of the paraventricular nucleus of the hypothalamus of an adrenalectomized (ADX) rat after incubation with antiserum to VP (A) and to CRF (B). A and B show thin (5 μm) adjacent sections. Numerous VP (AVP)- and CRF-immunoreactive cell profiles are seen, several of which (compare corresponding arrows in A and B) belong to the same cells. For orientation purposes identical blood vessels have been marked (V). (x700.)